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# Kinetic and Structural Characterization of Adsorption-induced Unfolding of Bovine $\alpha$ -Lactalbumin\*

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**Conformational changes of bovine  $\alpha$ -lactalbumin induced by adsorption on a hydrophobic interface are studied by fluorescence and circular dichroism spectroscopy. Adsorption of bovine  $\alpha$ -lactalbumin on hydrophobic polystyrene nanospheres induces a non-native state of the protein, which is characterized by preserved secondary structure, lost tertiary structure, and release of calcium. This partially denatured state therefore resembles a molten globule state, which is an intermediate in the folding of bovine  $\alpha$ -lactalbumin. Stopped-flow fluorescence spectroscopy reveals two kinetic phases during adsorption with rate constants  $k_1 \sim 50 \text{ s}^{-1}$  and  $k_2 \sim 8 \text{ s}^{-1}$ . The rate of partial unfolding is remarkably fast and even faster than unfolding induced by the addition of 5.4 M guanidinium hydrochloride to native  $\alpha$ -lactalbumin. The large unfolding rates exclude the possibility that unfolding of bovine  $\alpha$ -lactalbumin to the intermediate state occurs before adsorption takes place. Stopped-flow fluorescence anisotropy experiments show that adsorption of bovine  $\alpha$ -lactalbumin on polystyrene nanospheres occurs within the dead time (15 ms) of the experiment. This shows that the kinetic processes as determined by stopped-flow fluorescence spectroscopy are not affected by diffusion or association processes but are solely caused by unfolding of bovine  $\alpha$ -lactalbumin induced by adsorption on the polystyrene surface. A scheme is presented that incorporates the results obtained and describes the adsorption of bovine  $\alpha$ -lactalbumin.**

Adsorption of proteins on solid/liquid interfaces is a generally occurring phenomenon both in nature and in man-made systems. It is well recognized that proteins undergo conformational changes upon adsorption on solid/liquid interfaces (1–3). Because unfolding of proteins can have a large effect on their function or properties, it is important to increase the knowledge about protein adsorption and about the resulting conformational changes. It is necessary to study not only the conformation of a protein in the adsorbed state but also the kinetics of the adsorption-induced conformational changes. This knowledge will help in controlling wanted or unwanted conformational changes of adsorbed proteins.

In this work we focus on the kinetics of adsorption-induced

conformational changes of bovine  $\alpha$ -lactalbumin (BLA)<sup>1</sup> upon interaction with colloidal polystyrene nanospheres. Detailed information on adsorption-induced conformational changes and especially on the kinetics of adsorption-induced conformational changes is sparse. An important reason for this is the experimental difficulty of the presence of an adsorbent, which is usually a solid phase. A few routes have been designed to remove this difficulty. A macroscopic solid/water interface can be used in combination with a reflective technique like ellipsometry or total internal reflection fluorescence (4, 5). In other cases a microscopic system has been used that consists of small particles with diameters ranging from nanometers to micrometers in size (6, 7). The latter system has the advantage of providing a large interface, however, light scattering and light absorbance often interfere with several spectroscopic techniques. Here, we use a suspension of polystyrene nanospheres with a diameter of  $\sim 50 \text{ nm}$ . The large surface area per gram of polystyrene and therefore the high binding capacity allows a reduction of the nanosphere concentration. As a result, light scattering and absorption are reduced and thus the use of spectroscopic methods like stopped-flow fluorescence is feasible.

Bovine  $\alpha$ -lactalbumin is a small protein (14 kDa), containing four tryptophan residues and four disulfide bonds. It can bind calcium, which plays an important role in the stability and folding behavior of the protein (8). BLA is chosen in this study for several reasons. First, the structure, stability, and folding behavior of BLA have been thoroughly studied (9–11). Second, BLA is a protein that is applied at solid/water interfaces, for example in food applications. Finally, the folding pathway of BLA includes a stable intermediate, the so-called molten globule state, which has been studied in detail previously (12–14).

Isothermal titration calorimetry and intrinsic fluorescence spectroscopy have indicated denaturation of BLA upon adsorption on hydrophobic interfaces (15, 16). A study of Banuelos and Muga (17) shows that BLA binds to lipid bilayers in a molten globule-like conformation. Recently some studies (18–20) emerged that describe the kinetics of conformational changes induced by adsorption on an interface or interaction with lipid vesicles. Unfortunately, still little is known about the altered conformation in the adsorbed state and about the kinetics of the conformational changes that take place upon adsorption. Further knowledge on these matters is of particular relevance for the understanding of protein-membrane interactions and in the field of synthetic biomaterials used for medical applications.

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<sup>1</sup> The abbreviations used are: BLA, bovine  $\alpha$ -lactalbumin; GdnHCl, guanidinium hydrochloride; MG, molten globule; CD, circular dichroism; ICP-OES, inductively coupled plasma-optical emission spectrometry.

Regarding protein-membrane interactions, it is hypothesized that, when proteins are translocated across the membrane, they must be in a non-native or molten globule (MG) state (21). Previous work has indicated that, upon interaction with model membranes, BLA indeed can adopt an MG state (17). However, the interaction of proteins with model membranes can occur via two mechanisms: (a) adsorption on the membrane interface and (b) combined adsorption and insertion/penetration into the membrane bilayer (22). In the study presented here, BLA is adsorbed on a solid interface, so insertion or penetration cannot occur. The results show that adsorption itself suffices to cause a conformational change of BLA to an adsorbed state with molten globule properties.

Although the polystyrene nanospheres used are not a biological system, they are, at the moment, the only solid/liquid interface system that allows the study of the effect of adsorption (excluding penetration) on protein conformation with kinetic spectroscopic methods. We believe that the results obtained widen the biological view of protein-membrane interactions.

Furthermore, our study is relevant in the field of synthetic biomaterials. Synthetic polymer biomaterials are used in medical applications, for example as implants in the human body. Contact between these biomaterials and tissue or blood gives rise to all kinds of complex and mostly unwanted phenomena. Upon implantation of foreign material in the human body, proteins adsorb on the biomaterial. Knowledge of this process is relevant in the search for new and better biomaterials that can reduce adverse reactions of the human body.

In this report, the adsorption-induced unfolding of bovine  $\alpha$ -lactalbumin (BLA) is studied by intrinsic fluorescence and circular dichroism (CD) spectroscopy. Both the nature and the kinetics of conformational changes of BLA upon adsorption on a polystyrene/water interface are characterized. Several theoretical models exist that describe the kinetics of adsorption as well as the kinetics of adsorption-induced conformational changes (2). The experimental difficulty of discriminating between both kinetic processes has hampered the experimental verification of these models. Here we show that the kinetics of adsorption and the kinetics of adsorption-induced conformational changes can be distinguished. In fact, it is demonstrated that the kinetics of both the diffusion and the adsorption processes do not need to be taken into account to enable analysis of the kinetics of the conformational change of the adsorbed protein as observed in stopped-flow experiments.

## EXPERIMENTAL PROCEDURES

### Materials

Bovine  $\alpha$ -lactalbumin (L-5385, Sigma Chemical Co.) was used without further purification. Polystyrene nanospheres, supplied as a colloidal suspension in water, were obtained from Polymer Laboratories (Heerlen, The Netherlands). Nanosphere suspensions were diluted with buffer before use. All experiments were done in a 10 mM Tris/HCl buffer of pH 7.50 containing 1 mM  $\text{CaCl}_2$ . The added calcium ensures a constant calcium concentration during the adsorption and unfolding experiments, because calcium has a drastic effect on the stability and folding behavior of BLA. Nanopure water was used in all experiments (Sybron Barnstead NANOpure II).

### Methods

**Dynamic Light Scattering**—Dynamic light scattering was performed on an experimental setup composed of a Lexal 150-milliwatt laser operating at a wavelength of 514.5 nm, a photomultiplier, and a personal computer containing an ALV5000 correlation card. Scattered intensities were recorded at a 90° scattering angle and at 20 °C. A total of 10 measurements of each 10 s were averaged. The data were analyzed by the ALV5000 software. Samples were diluted in the buffer mentioned above. The nanosphere concentration ranged from 0.05 to 0.25 mg/ml.

**Electrophoretic Mobility**—The electrophoretic mobility of the polystyrene nanospheres (3 mg/ml) suspended in a 10 mM Tris/HCl buffer at

pH 7.5 with 1 mM  $\text{CaCl}_2$  was determined by microelectrophoresis (Malvern Zetasizer III). The Malvern computer program was used to calculate the zeta-potential from the electrophoretic mobility.

**Adsorption Isotherm**—The adsorption isotherm was determined according to the depletion method. The amount of free protein after adsorption was measured spectrophotometrically using a molar extinction coefficient of  $28,540 \text{ M}^{-1} \text{ cm}^{-1}$  for BLA at 280 nm. The polystyrene nanospheres were separated from free  $\alpha$ -lactalbumin by using Centricon 100 ultrafiltration devices (Millipore Corp.). Protein adsorption on the filter was checked and found to be negligible. The filter retained more than 99.8% of the polystyrene nanospheres. In a typical adsorption experiment 2.5 ml of protein solution was added to 2.5 ml of nanosphere suspension in a 10-ml polysulfone centrifuge tube (Nalgene, Oak Ridge, TN). The tubes were then placed on a rocking table and gently shaken at room temperature. Adsorption isotherms were determined after 30 min of adsorption and after 24 h of adsorption. Different protein concentrations were used to construct an adsorption isotherm. The resulting adsorption isotherm was used to determine the molar protein-to-nanosphere ratio ( $n$ ) that gave maximal protein monolayer adsorption and minimal free protein. This ratio was used in all adsorption experiments, unless mentioned otherwise. For determination of the desorbed amount of BLA, the nanospheres covered with BLA where separated from the solution with Centricon 100 ultrafiltration devices. Separation was done at 4 °C without calcium or at 20 °C and in the presence of either 10 or 100 mM  $\text{CaCl}_2$ . Subsequently the concentration of BLA in solution was determined spectrophotometrically.

**Determination of the  $K_d$  for Calcium Binding on the Nanospheres**—Polystyrene nanospheres (80 nm) were incubated with different amounts of  $\text{CaCl}_2$  (0, 5, 10, 40, 100, 200, and 400  $\mu\text{M}$ ) for 20 h at room temperature in a calcium-free 10 mM Tris/HCl buffer at pH 7.5. The nanospheres covered with calcium were separated from the calcium containing solution by ultrafiltration (Amicon, YM50 membrane, Millipore Corp.). The filtrate was analyzed for calcium content by inductively coupled plasma-optical emission spectrometry (ICP-OES). The amount of adsorbed calcium was calculated from the original amount and the amount of free calcium after filtration. An adsorption isotherm was constructed and fitted with a Langmuir isotherm:  $[\text{Ca}]_{\text{adsorbed}} = (B \cdot [\text{Ca}]_{\text{free}}) / (K_d + [\text{Ca}]_{\text{free}})$ . In this equation,  $B$  is the plateau value of adsorption, and  $K_d$  is the dissociation constant. The concentration of  $\text{CaCl}_2$  stock solutions was determined with ICP-OES.

**Fluorescence Spectroscopy**—Fluorescence spectra and time-dependent fluorescence traces were measured in a quartz cuvette (10  $\times$  4 mm) on a Fluorolog 2 (SPEX). The temperature of the cuvette holder was maintained at 20 °C with a water bath in all experiments. Excitation was at 300 nm, with excitation slits at 3 nm and emission slits at 5 nm. A blank, containing all components except protein, was subtracted from each sample. Nanosphere concentrations ranged up to 0.05 mg/ml. The total absorbance of the samples at 300 nm was kept below 0.1 to minimize the inner filter effect. To ensure a molar protein to nanosphere ratio of 500, the maximum protein concentration was about 0.5  $\mu\text{M}$ . In the manual mixing adsorption experiments the nanospheres were added to the stirred protein solution with an automatic pipette. Typically, an amount of 100  $\mu\text{l}$  of nanosphere suspension was added to 1.5 ml of protein solution. In time-dependent fluorescence measurements, care was taken to avoid photobleaching of the fluorophore, by ensuring discontinuous illumination with enough (dark) time between data points.

**Fluorescence Anisotropy**—Fluorescence anisotropy was measured on a home-built fluorometer equipped with two photomultipliers arranged in T-format (Thorn EMI 9863QA/350, operating in photon-counting detection mode). The light was generated by a 150-watt short arc xenon lamp, and the excitation wavelength of 300 nm was selected in a monochromator (Bausch and Lomb) with a bandpass of 3.2 nm. Polarizers were used in both the excitation light path (rotatable Glan Taylor polarizer) and the emission light path (Polaroid, sheet). The emission light was selected with a 335-nm cut off filter and a UG1 filter (Schott). A blank measurement, containing all components except BLA, was subtracted from each sample, and five measurements were averaged for each sample.

**Stopped-flow Fluorescence Spectroscopy**—Stopped-flow fluorescence and stopped-flow fluorescence anisotropy were measured on a BioLogic SFM4 equipped with a 2- $\times$  2-mm cuvette (FC-20). Excitation was at 300 nm, and excitation slits were set at 0.5 mm, resulting in a bandpass of 4 nm. A cut-off filter of 335 nm (335FG01-25, Andover Corp.) and a 330wb60 bandpass filter (XF3000-25, Omega Optical Inc.) were used together to select the emission light. The temperature was kept constant at 20 °C with a thermostatic water bath. Ten measurements were averaged for each sample. In a typical run 150  $\mu\text{l}$  of a 5  $\mu\text{M}$  protein



solution was mixed with 150  $\mu$ l of a 10 nM nanosphere suspension in 75 ms. A flow speed of 4 ml/s resulted in a dead time of 15 ms. Faster flow speeds were not used, because this resulted in signal distortion and bad reproducibility, probably due to cavitation. Results from the stopped-flow fluorescence experiments were analyzed and fitted with the Padé-Laplace algorithm in the Bio-Kine software (Bio-Logic), using a minimum number of exponential phases. Prior to fitting the data, the signal of the polystyrene nanospheres was subtracted from the sample, and the time axis was adjusted, resulting in a shift of the first data point to  $t = 0$  s. A fit was evaluated and judged to be correct when random noise around a horizontal line centered at zero was observed for the residual values. Stopped-flow fluorescence anisotropy was measured with a single photomultiplier and no polarizer in the emission light path. A similar set up has been reported recently in a study on protein folding in solution (23). In the anisotropy set-up the excitation light is modulated with a photoelastic modulator to give alternating horizontally and vertically polarized light with a frequency of 100 kHz. The synchronized electronics connected to the photomultiplier simultaneously record the fluorescence emission intensity at vertical excitation ( $I_v$ ) and at horizontal excitation ( $I_h$ ). A blank, containing all components except BLA, was subtracted from the sample resulting in the corrected values  $I_v^*$  and  $I_h^*$ . The anisotropy ( $A$ ) for each data point was then calculated in a spreadsheet application according to the equation:  $A = (I_v^* - I_h^*)/(I_v^* + I_h^*/2)$ .

**Circular Dichroism Spectroscopy**—CD measurements were performed on a Jasco J-715 spectropolarimeter, equipped with a Peltier temperature control system set at 20 °C. Calibration was performed with an ammonium D-10-camphorsulfonate solution in nanopure water for which the concentration was checked spectrophotometrically. Typical protein concentrations used were 1.5  $\mu$ M in a 0.1-cm cuvette for far-UV CD and 4.5  $\mu$ M in a 1-cm cuvette for near-UV CD measurements. Eight scans were averaged for each sample. The CD spectra of BLA adsorbed on the nanospheres were averaged from 160 and 128 scans for the far-UV region and near-UV region, respectively. In the case of adsorbed BLA, sample and blank spectra were recorded alternately in sets of 32 scans, to avoid disturbances due to instrumental drift. For each set of 32 scans, a fresh sample of BLA adsorbed on the nanospheres was used. The response time was 1 s, and the spectral bandwidth was 1.0 nm. Before analysis of the spectra, a blank, containing all components except BLA, was subtracted from the sample.

## RESULTS

**Characterization of the Polystyrene Nanospheres**—The radius of the nanospheres diluted in a 10 mM Tris/HCl buffer at pH 7.50 with 1 mM  $\text{CaCl}_2$  is  $47 \pm 1$  nm as determined by dynamic light scattering. Based on this value a surface area of  $122 \pm 3$  m<sup>2</sup>/g is calculated. Investigation by transmission electron microscopy shows that the nanospheres are spherical and that possible irregularities at the surface are smaller than 2 nm in size (results not shown). The zeta-potential for the nanospheres as determined by electrophoretic mobility is  $-38 \pm 1$  mV in a 10 mM Tris/HCl buffer with 1 mM  $\text{CaCl}_2$  at pH 7.5. This value indicates a negative surface charge of the polystyrene nanospheres in this particular buffer. The diameter of the nanospheres after adsorption of a monolayer of BLA is  $49 \pm 1$  nm and remains the same during 24 h. No aggregation of the nanospheres occurs after adsorption of BLA.

**Adsorption Isotherm**—The adsorption isotherm of BLA on polystyrene nanospheres is shown in Fig. 1. The plateau value of  $3 \pm 0.2$  mg/m<sup>2</sup> agrees well with previously reported values. Adsorption of BLA on a platinum surface resulted in a value of 2.9 mg/m<sup>2</sup> (24), and a value of 3.3 mg/m<sup>2</sup> was found for adsorption of BLA on a hydrophobic silicon surface (25). The steep part in the region of low BLA concentration is an indication for high affinity behavior. In the initial, steep part of the adsorption isotherm, all protein present in the system is adsorbed on the surface of the polystyrene nanospheres and virtually no free BLA is present in solution. If a molar nanosphere-to-protein ratio of 1:500 is chosen, then all BLA added is adsorbed and there is no free BLA present in the solution. This ratio corresponds with the region in the adsorption isotherm of the initial steep part, before a coverage of 3 mg/m<sup>2</sup> is reached. This ratio is used in the adsorption experiments presented below,

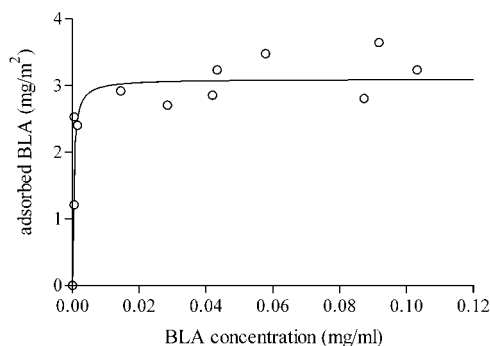


FIG. 1. Adsorption isotherm of bovine  $\alpha$ -lactalbumin on polystyrene nanospheres in a 10 mM Tris/HCl buffer with 1 mM  $\text{CaCl}_2$  at pH 7.5 after 24 h of adsorption. Circles, data; line, fit. Adsorption isotherms determined after an adsorption time of 30 min and after an adsorption time of 24 h are identical.

assuming that all added protein is adsorbed on the nanospheres.

The polystyrene nanospheres and the BLA molecules both have a net negative charge under the conditions used. The fact that adsorption occurs means that the electrostatic repulsion has to be overcome. Hydrophobic interactions therefore contribute the most to the adsorption process, as was found for the adsorption of BLA on hydrophobic interfaces (15, 26). However, minor contributions of electrostatic interactions cannot be excluded. They may play a role in the orientation of the adsorbed BLA molecules (see "Discussion").

Desorption of BLA from the polystyrene nanospheres is tested with different methods. Washing the BLA-covered nanospheres with buffer does not result in protein desorption, even after 24 h. Hardly any desorption of BLA from the polystyrene nanospheres takes place when the calcium concentration is increased. Only 12% of the adsorbed BLA molecules is desorbed when the calcium concentration is increased to 10 mM, whereas only 14% is desorbed at a calcium concentration of 100 mM after 24 h of incubation. In an attempt to decrease the hydrophobic interactions and enable BLA desorption the temperature was decreased. However, storage during 24 h at 4 °C and in the presence of 1 mM  $\text{CaCl}_2$  gave no desorption of BLA molecules.

**Adsorption-induced Conformational Changes of BLA**—Fluorescence spectra of BLA in solution and of BLA adsorbed on nanospheres after 5 and 75 min of adsorption are shown in Fig. 2. The spectrum of BLA in solution shows a fluorescence emission maximum at 325 nm, which indicates that the Trp residues are buried in the hydrophobic interior of the protein. The adsorbed BLA shows a maximum at 335 nm and an intensity increase at maximum wavelength of about 100% compared with the spectrum of BLA in solution. The shift of the fluorescence maximum indicates that the tryptophan residues of BLA in the adsorbed state are in a more exposed environment. The intensity increase is related to a reduction in quenching that results from a different position of the tryptophan residues compared with their original position in the three-dimensional structure of native BLA. For native BLA it is suggested that energy transfer occurs from Trp<sup>26</sup> and Trp<sup>104</sup> to Trp<sup>60</sup> and, furthermore, the fluorescence is quenched by two disulfide bonds near Trp<sup>60</sup> (Cys<sup>73</sup>-Cys<sup>91</sup> and Cys<sup>61</sup>-Cys<sup>77</sup>) (27). It has also been observed that unfolding of BLA results in a red shift of the fluorescence emission maximum and an accompanied increase in fluorescence intensity (28). Complete unfolding of  $\alpha$ -lactalbumin by 5.4 M guanidinium hydrochloride results in a fluorescence emission maximum of 350 nm indicating complete exposure of the Trp residues (results not shown). A measured fluorescence emission maximum of 335 nm would thus indicate that the adsorption-induced unfolding is not complete, leaving

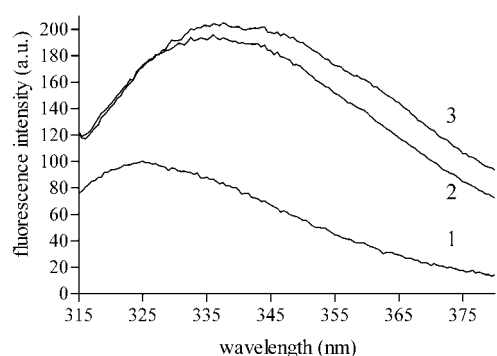


FIG. 2. Fluorescence emission spectra of 0.5  $\mu$ M BLA in solution (1) and of 0.5  $\mu$ M BLA adsorbed on 0.05 mg/ml polystyrene nanospheres after 5 (2) and 75 (3) min of adsorption in a 10 mM Tris/HCl buffer with 1 mM  $\text{CaCl}_2$  at pH 7.5 and at 20  $^\circ\text{C}$ . The fluorescence intensity is normalized to a value of 100 for the maximum at 325 nm for BLA in solution. The excitation wavelength was 300 nm.

one or more Trp residues in the hydrophobic core of BLA.

Circular dichroism spectra of free and of adsorbed BLA in the far-UV region and in the near-UV region are shown in Figs. 3 (A and B, respectively). The far-UV CD spectrum of adsorbed BLA shows that a considerable amount of secondary structure remains after adsorption. Although the spectrum of adsorbed BLA contains a lot of noise, the characteristic ellipticity minimum at 208 nm is visible. This indicates that the secondary structure of native BLA and of adsorbed BLA is similar. The near-UV CD spectra show a clear difference between native BLA and adsorbed BLA. The spectrum of BLA in solution has a characteristic profile that includes a broad Tyr minimum around 270 nm and a local Trp minimum at 297 nm. The spectrum of adsorbed BLA does not show these characteristics and has a decreased molar ellipticity over the entire near-UV CD spectrum, which indicates disruption of tertiary structure elements. The latter is in agreement with the changes seen in the fluorescence emission spectrum upon protein adsorption (see above).

The difficulty of working with polystyrene nanospheres in suspensions emerges in particular when working with far-UV light in CD spectroscopy (Fig. 3A). The CD measurements in the far-UV region were performed with a nanosphere concentration that results in a UV absorption of 0.5 at 220 nm in a 0.1-cm light path cuvette, whereas in the near-UV a concentration of nanospheres is used, which results in a UV absorption of 0.5 at 280 nm in a 1-cm light path cuvette. The protein concentration was adjusted to obtain a ratio of 500 protein molecules to 1 nanosphere particle, as is used throughout this work. The loss of light intensity in the CD measurements of the adsorbed protein, which is due to both light scattering of the polystyrene nanospheres and light absorption of the polystyrene molecules in the nanospheres, leaves only half of the original intensity for the characterization of the protein structure. Consequently, a significant increase of noise in the data is observed. Despite the difference in the noise level between the spectra shown in Fig. 3, it is clear that upon BLA adsorption a disruption of tertiary structure elements occurs without significant loss of secondary structure.

**Kinetics of Adsorption-induced Conformational Changes—**Stopped-flow fluorescence spectroscopy is used to investigate the kinetics of the adsorption process. To our knowledge, we demonstrate here for the first time protein adsorption on solid spheres in the stopped-flow technique. The small size of the nanospheres allows the use of this technique, without the risk of blocking the small mixing channels in a stopped-flow apparatus. A typical example of a stopped-flow trace is shown in Fig. 4. The experiments are performed with different BLA/nano-

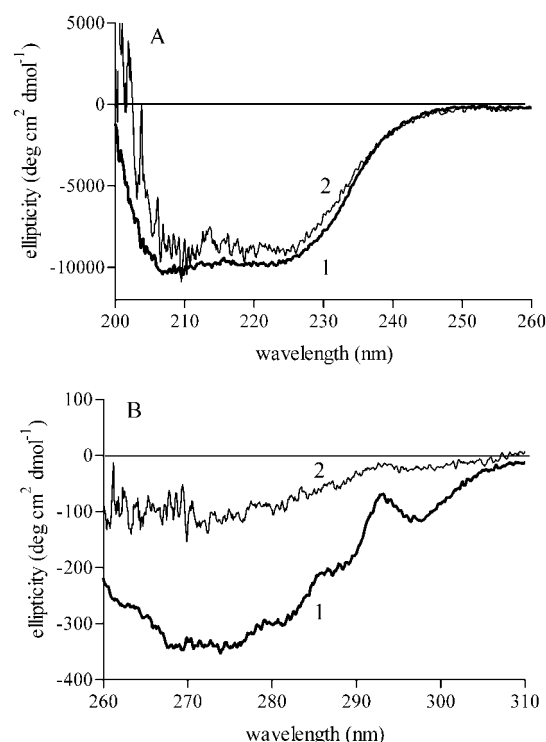


FIG. 3. Far-UV (A) and near-UV (B) CD spectra of BLA in solution (1, thick line) and of BLA adsorbed on polystyrene nanospheres (2, thin line) in a 10 mM Tris/HCl buffer with 1 mM  $\text{CaCl}_2$  at pH 7.5 and at 20  $^\circ\text{C}$ . The concentrations used for far-UV CD spectra are: 1.5  $\mu$ M BLA and 3 nM nanospheres, in a 1-mm cuvette. The concentrations used for near-UV CD spectra are: 4.5  $\mu$ M BLA and 10 nM nanospheres, in a 10-mm cuvette.

sphere ratios ranging from about 1100 to 160 BLA molecules for each nanosphere. This ratio covers the steep part of the adsorption isotherm as described before (Fig. 1). Below a ratio of 500 all BLA molecules are adsorbed on the polystyrene nanospheres and no free BLA is left in solution.

The stopped-flow traces are fitted to a double-exponential equation, except for the measurement at the lowest BLA concentration where one exponential term suffices (Table I). The rate constants observed are between 38 and 74  $\text{s}^{-1}$  for the fast phase ( $k_1$ ) and between 6 and 10  $\text{s}^{-1}$  for the slower phase ( $k_2$ ). Only  $k_1$  depends on the BLA concentration. The major phase is the fastest phase that accounts for 30–35% of the total fluorescence change. About 50–70% of the fluorescence intensity increase occurs in the dead time of 15 ms ( $f_d$  in Table I). However, the change of amplitude within the dead time is roughly as expected based on  $k_1$  and currently does not suggest the presence of another faster phase.

Manual mixing experiments reveal a third phase that is responsible for 10% of fluorescence intensity increase, which occurs between 1 and 30 min after mixing BLA and the nanospheres (Fig. 5A). The increase in fluorescence intensity is accompanied by a shift in the fluorescence maximum from 326 to 336 nm within the first 15 s (Fig. 5A), the minimum time needed to obtain an emission wavelength scan. The gradual increase that occurs after the initial fluorescence burst depends on the emission wavelength (Fig. 6). At low emission wavelength (320 nm), the fluorescence intensity is constant between 12 s and 75 min after mixing BLA and the nanospheres. However, at higher emission wavelengths (340, 360, and 380 nm) the gradual increase in fluorescence intensity becomes more pronounced, which indicates the involvement of Trp residues in an exposed environment. The gradual increase in fluorescence intensity suggests slow structural rearrangements of adsorbed

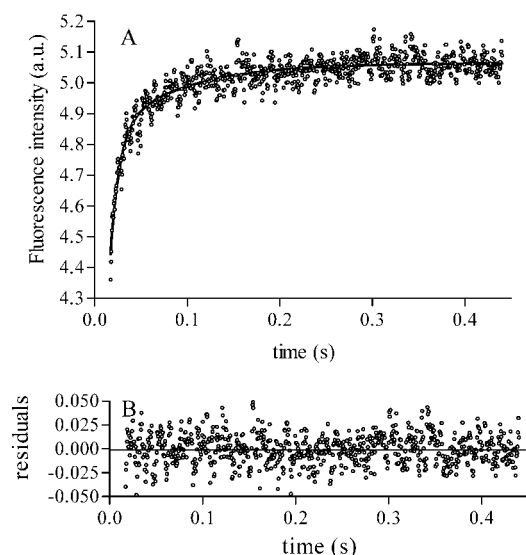


FIG. 4. **Stopped-flow fluorescence trace (circles) of BLA adsorption on polystyrene nanospheres in a 10 mM Tris/HCl buffer with 1 mM  $\text{CaCl}_2$  at pH 7.5 and at 20 °C.** A, the BLA concentration was 4.0  $\mu\text{M}$  and the nanosphere concentration was 5.0 nM. The fluorescence trace was fitted (line) with a double-exponential function. Rate constants resulting from the fit are:  $k_1 = 74 \text{ s}^{-1}$  and  $k_2 = 7.5 \text{ s}^{-1}$ . B, residuals of the fit.

BLA molecules. The data at different emission wavelengths, representing fluorescence changes between 12 s and 75 min, were fitted separately to a single-exponential function (Table II). The rate constants for this phase are about  $1 \times 10^{-3} \text{ s}^{-1}$ . The near-UV CD trace of the manual mixing experiments shows that the change in conformation occurs within 10 s after the addition of the nanospheres (Fig. 5B). The ellipticity at 275 nm increases from the native value of  $-350 \text{ deg cm}^2 \text{ dmol}^{-1}$  to the steady-state value of  $-150 \text{ deg cm}^2 \text{ dmol}^{-1}$  within 10 s, which is the time necessary to open the sample chamber, add the nanospheres, mix, and close the sample chamber again. Because of the low sensitivity of the CD method, it is not possible to track small changes in the signal that could occur after the initial increase of the signal.

In summary, the changes in fluorescence emission intensity induced by adsorption of BLA on the polystyrene nanospheres show the existence of three phases. The first phase has a rate constant of 38 to  $74 \text{ s}^{-1}$  depending on the BLA concentration. This phase contributes 22–37% to the total fluorescence emission intensity increase in a stopped-flow trace and is also responsible for the 50–70% increase in fluorescence emission intensity during the dead time of the experiment. The phase is associated with the major unfolding of BLA in its native state to an intermediate folding state, which is adsorbed on the polystyrene nanospheres and has molten globule-like characteristics. Thus a state with preserved secondary structure and disrupted tertiary structure already exists shortly after adsorption of BLA on the nanospheres. The second and third, slower phases are responsible for about 10% of the fluorescence signal change and have rate constants of between 6 to  $10 \text{ s}^{-1}$  ( $k_2$ ) and of about  $0.001 \text{ s}^{-1}$  ( $k_3$ ), respectively.

**Diffusion and Association Do Not Affect the Observed Stopped-flow Fluorescence Traces**—It has been recognized that three processes must play an important role during the complex kinetics of protein adsorption and subsequent unfolding (2). First, the BLA molecules have to find their way to the surface of the polystyrene nanospheres. This process is governed by the kinetics of mass transport: Both convective transport and diffusion play an important role. Because mixing in the stopped-flow apparatus is virtually ideal, we will not con-

sider convective transport. Second, the protein molecules that are in the vicinity of the interface should adsorb on the interface. The rate of this association process is governed by several factors, including the sticking probability and the coverage of the adsorbent surface. The third process involves conformational changes of the adsorbing protein, which is the subject of this paper. The previously discussed stopped-flow fluorescence traces that led to the conclusions about the conformational changes of adsorbing BLA molecules might be interwoven with the diffusion controlled process and/or with the association-controlled process. These processes would become visible in the stopped-flow fluorescence traces when the conformational change, which leads to increased fluorescence intensity, would be much faster than the rate of diffusion or association. The possibility of diffusion-limited processes affecting the stopped-flow fluorescence traces in our experiments should be addressed, because especially the size of the polystyrene nanospheres is large compared with the size of a BLA molecule, and because their concentration is much lower than for example the GdnHCl concentration in a common stopped-flow unfolding experiment.

In case diffusion is a limiting factor in the kinetic process, its contribution can be estimated. The maximum rate of adsorption of a protein on a sphere for a diffusion-limited process can be calculated by using Equation 1 (29). The equation is derived from a calculation of the average flow of protein molecules to a sphere,

$$k_{\text{diff}} = 4\pi N_A R_s D_p \quad (\text{Eq. 1})$$

where  $k_{\text{diff}}$  is the diffusion-limited association rate constant ( $\text{M}^{-1} \text{ s}^{-1}$ ),  $N_A$  is Avogadro's number ( $\text{mol}^{-1}$ ),  $R_s$  is the radius of the sphere (meters), and  $D_p$  is the diffusion coefficient of the protein ( $\text{m}^2/\text{s}$ ). For the adsorption of BLA on polystyrene nanospheres (radius, 24 nm; diffusion coefficient,  $1.06 \times 10^{-10} \text{ m}^2/\text{s}$ ; as determined by dynamic light scattering) a diffusion-limited association rate constant of  $1.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$  is calculated. For the protein concentrations used, diffusion-limited association rates between 15,000 and  $75,000 \text{ s}^{-1}$  are calculated. These rates are much higher than the ones found in the analysis of the stopped-flow fluorescence traces (Table I). Consequently, diffusion does not affect our stopped-flow data.

Equation 1 is based on the assumption that the concentration of protein in solution is constant, but this is not the case in our experiments. On the contrary, the protein concentration decreases and in some cases even drops to zero in the course of the adsorption process. As a result, the rate of diffusion would also decrease when more and more protein molecules are adsorbed and thus less BLA is present in solution. However, calculations show that, even when the protein concentration has dropped to 1% of its original value, the diffusion-limited rate is still much higher than the fastest process we observe.

As indicated, the rate of association of the proteins to the nanospheres is another factor that could affect the observed unfolding rates. Because the surface of the polystyrene nanospheres is increasingly covered by BLA molecules during adsorption, and because the protein concentration decreases during adsorption, it is expected that the association rate decreases in time. In our experiments a protein-to-particle ratio is used such that virtually all molecules adsorb and form a monolayer on the polystyrene surface. This means that the protein concentration in solution drops to almost zero and coverage of the surface increases to almost its maximum during adsorption. Unfortunately, whether an association-limited phase affects the interpretation of our stopped-flow fluorescence data cannot be easily predicted.



TABLE I

Fit results of the data obtained from stopped-flow fluorescence experiments of BLA adsorption on polystyrene nanospheres

The kinetic adsorption experiments were performed in a 10 mM Tris/HCl buffer of pH 7.5 with 1 mM  $\text{CaCl}_2$  at 20 °C. The excitation wavelength was 300 nm. The dead time was 15 ms;  $n$  is the molar ratio BLA/nanospheres;  $p_0$  is expressed as the total fluorescence amplitude (arbitrary units);  $f_1$ ,  $f_2$ , and  $f_d$  are the amplitudes of the correspondent phases, expressed as fraction of the total amplitude;  $f_d$  is the amplitude in the dead time.<sup>a</sup>

BLA	Nanosphere	$n$	$p_0$	$f_1^b$	$k_1^b$	$f_2^b$	$k_2^b$	$f_d^b$
$\mu\text{M}$	$\text{nM}$				$\text{s}^{-1}$		$\text{s}^{-1}$	
0.80	4.97	161	0.87	0.53	38			0.47
1.34	4.97	270	1.46	0.36	47	0.17	7.2	0.47
1.87	4.97	376	1.76	0.37	48	0.11	6.6	0.52
2.27	4.97	457	1.81	0.36	51	0.09	6.3	0.55
2.67	4.97	537	2.10	0.31	68	0.11	8.5	0.58
3.07	4.97	618	1.99	0.30	74	0.10	10.1	0.60
4.00	4.97	805	2.43	0.22	74	0.07	7.5	0.71
2.63	2.33	1129	0.85	0.37	55	0.12	6.3	0.51
2.63	3.33	790	1.17	0.37	61	0.11	8.2	0.52
2.63	4.67	563	1.69	0.33	55	0.09	7.5	0.58
2.63	5.67	464	1.75	0.35	51	0.09	6.3	0.56
2.63	6.67	394	2.19	0.30	67	0.12	8.9	0.58
2.63	7.67	343	2.25	0.30	58	0.07	7.0	0.63
2.63	10.00	263	3.04	0.25	67	0.08	9.7	0.67

<sup>a</sup> Stopped-flow fluorescence data are fitted according to  $I(t) = p_0 + p_1 e^{(-tk_1)} + p_2 e^{(-tk_2)}$ .

<sup>b</sup> The standard deviations are  $\pm 0.03$  for  $f$  values,  $\pm 3 \text{ s}^{-1}$  for  $k_1$ , and  $\pm 1 \text{ s}^{-1}$  for  $k_2$ . The standard deviations are based on the results of three separate fits on the time-dependent fluorescence trace. Each trace is the average of ten individual measurements.

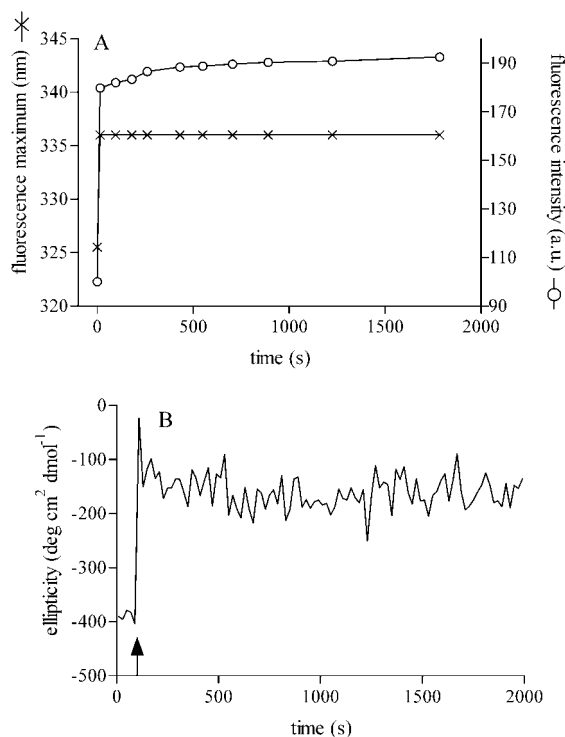


FIG. 5. Manual mixing experiments in which BLA is adsorbed onto polystyrene nanospheres in a 10 mM Tris/HCl buffer with 1 mM  $\text{CaCl}_2$  at pH 7.5 and at 20 °C. A, normalized fluorescence intensity at 330 nm (circles, right axis) and fluorescence spectral maximum (crosses, left axis) after addition of polystyrene nanospheres (1 nM) to a BLA (0.5  $\mu\text{M}$ ) solution at  $t = 0$  s. B, molar ellipticity at 275 nm of BLA (4.5  $\mu\text{M}$ ) adsorption on polystyrene nanospheres (10 nM). The arrow indicates the moment of addition of the nanospheres to the BLA solution. The initial peak after the addition of the nanospheres is caused by opening of the sample chamber during 10 s and should not be considered as relevant data.

To confirm that the measured fluorescence traces as shown in the previous section are not affected by diffusion and/or association processes, fluorescence anisotropy has been measured during the adsorption process. The idea is that adsorption of BLA on the relatively large nanospheres will drastically decrease the rotational correlation time of BLA and thus lead

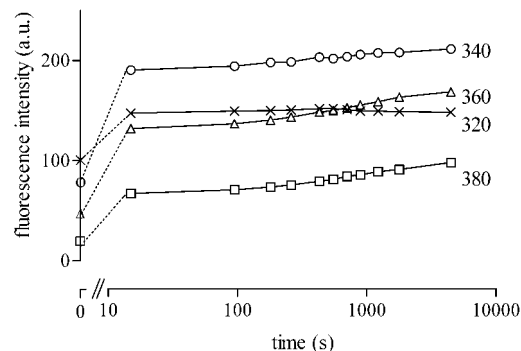


FIG. 6. Normalized fluorescence intensity at different emission wavelengths (320, 340, 360, and 380 nm) during the adsorption of BLA (0.5  $\mu\text{M}$ ) on polystyrene nanospheres (1 nM). Experiments were done in a 10 mM Tris/HCl buffer with 1 mM  $\text{CaCl}_2$  at pH 7.5 and at 20 °C. The manual mixing method resulted in a dead time of  $\sim 10$  s. Polystyrene nanospheres are added to a BLA solution at  $t = 0$  s. Excitation is at 300 nm. The fluorescence intensity of BLA in solution at an emission wavelength of 320 nm is normalized to 100. The other data points have been normalized correspondingly.

TABLE II

Fit results of the data obtained from manual mixing fluorescence experiments of BLA adsorption on polystyrene nanospheres

The adsorption experiments were performed in a 10 mM Tris/HCl buffer of pH 7.5 with 1 mM  $\text{CaCl}_2$  at 20 °C. The excitation wavelength was 300 nm. The dead time was about 10 s;  $\lambda_{\text{em}}$  is the emission wavelength;  $p_0$  is expressed as the total fluorescence amplitude (arbitrary units), the amplitude  $f_3$  is expressed as fraction of the total amplitude,  $k_3$  is a rate constant for unfolding.<sup>a</sup>

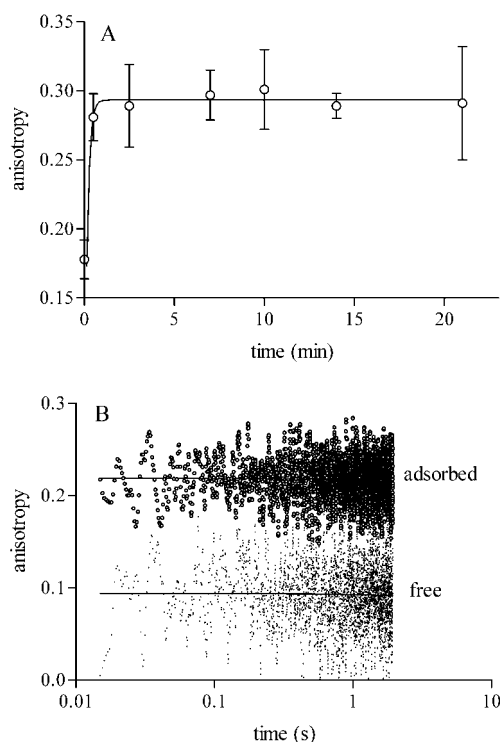
$\lambda_{\text{em}}$	$p_0$	$f_3^b$	$k_3^b$
$\text{nm}$			$\text{s}^{-1}$
320 <sup>c</sup>			
340	244	0.15	0.0016
360	408	0.28	0.0011
380	654	0.36	0.0010

<sup>a</sup> The time-dependent fluorescence data are fitted according to the equation  $I(t) = p_0 + p_3 e^{(-tk_3)}$ .

<sup>b</sup> Standard deviations are  $\pm 0.03$  for  $f$  values and  $\pm 0.0002 \text{ s}^{-1}$  for  $k_3$  based on two separate experiments.

<sup>c</sup> No fit was made because the data yielded a straight, horizontal line.

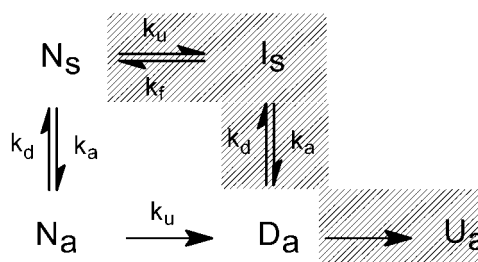
to an increase of its fluorescence anisotropy. A calculation based on the Perrin equation shows the relation between the fluorescence anisotropy ( $r$ ) and the rotational mobility of the fluorophore (Equation 2),



**FIG. 7. Time-dependent fluorescence anisotropy during adsorption of BLA on polystyrene nanospheres determined by a manual mixing experiment (A) and by a stopped-flow experiment (B).** The experiments were performed in a 10 mM Tris/HCl buffer with 1 mM  $\text{CaCl}_2$  at pH 7.5 and at 20 °C. The BLA concentration was 0.5  $\mu\text{M}$  and the nanosphere concentration was 1 nM in the manual mixing experiment, whereas the BLA concentration was 4.5  $\mu\text{M}$  and the nanosphere concentration was 10 nM in the stopped-flow experiment. The polystyrene nanospheres were added to the BLA solution at  $t = 0$ . The stopped-flow traces were fitted with a straight line, which resulted in anisotropy values of 0.094 for BLA free in solution and of 0.219 for BLA adsorbed on polystyrene nanospheres.

$$\frac{I_0}{r} = 1 + \frac{\tau}{\theta} \quad (\text{Eq. 2})$$

where  $r_0$  is the fundamental anisotropy of the fluorophore,  $r$  is the measured anisotropy,  $\tau$  is the fluorescence lifetime (seconds), and  $\phi$  is the rotational correlation time (seconds). The fluorescence lifetime of BLA is about 2.6 ns, and the fundamental anisotropy of tryptophan is about 0.30 at an excitation wavelength of 300 nm (30). The rotational correlation time for both BLA and nanospheres can be estimated based on their radius and on the assumption of both being spherical particles. This yields  $\phi$  values of 5 ns for BLA and of about 1  $\mu\text{s}$  for the nanospheres. Using Equation 2, the anisotropy of free and adsorbed BLA is predicted to be 0.20 and 0.30, respectively. The intrinsic fluorescence anisotropy is measured for the adsorption of BLA on polystyrene nanospheres, both in manual mixing experiments and in stopped-flow experiments. The results show that the anisotropy of free BLA increases from the value of 0.18 for BLA in solution to a steady-state value of 0.29 after addition of the nanospheres within the dead time of the manual mixing experiment, which is about 1 min (Fig. 7A). In a stopped-flow experiment, the anisotropy of free BLA mixed with buffer is 0.09 and remains constant, which is not surprising because no change in the rotational correlation time of the free BLA is anticipated (Fig. 7B). After mixing BLA with the nanospheres, the anisotropy is 0.22 and it remains constant during the 2-s duration of the stopped-flow experiment. Although the anisotropy measured by the manual mixing experiments agrees very well with the predicted values, those ex-



**FIG. 8. Schematic model for adsorption-induced unfolding of bovine  $\alpha$ -lactalbumin on a polystyrene interface.**  $N$  is the native state,  $I$  is the intermediate state (molten globule state),  $U$  is the completely unfolded state, and  $D$  is a denatured state, which strongly resembles the molten globule state of BLA in solution. The subscript 's' indicates the species in solution, and subscript 'a' indicates adsorbed species. The folding and unfolding rate constants  $k_f$  and  $k_u$  are shown, as well as the adsorption rate constant  $k_a$  and desorption rate constant  $k_d$ . The shaded areas indicate parts of the scheme that do not play a significant role in the adsorption of BLA, as shown by the results presented in this report (see text for further details).

tracted from the stopped-flow experiments do not. However, the increase in anisotropy upon BLA adsorption is nearly equal (*i.e.* about 0.12) for both experiments. Slight deviations in the wavelength and the bandpass of the excitation light between the manual mixing experiment and the stopped-flow experiment most likely cause the observed differences in the anisotropy values. The anisotropy of proteins containing tryptophan residues shows large differences upon altering the excitation wavelength between 295 and 300 nm (30).

Despite the differences in the absolute values of anisotropy obtained from manual mixing and stopped-flow experiments, the stopped-flow experiment clearly indicates an increased rotational correlation time upon adsorption of BLA on the nanospheres. The increase occurs within the dead time of the experiment, indicating that BLA is adsorbed within 15 ms. Consequently, the stopped-flow fluorescence traces are not affected by diffusion or association processes, but instead can be completely attributed to the unfolding of BLA adsorbed on the polystyrene nanospheres. The absorption and unfolding processes that play a role during adsorption of BLA on the polystyrene nanospheres are presented in a scheme, which will be discussed under "Discussion" (Fig. 8).

Because removal of calcium from BLA facilitates the formation of the molten globule state, we tested whether adsorbed BLA still contains calcium. Besides adsorbing proteins, we found that the nanospheres also adsorb calcium ions. Hence, it is not possible to detect free calcium ions in solution that are released from adsorbed BLA molecules. We determined the  $K_d$  for the interaction of calcium ions with the polystyrene nanospheres (Fig. 9). A Langmuir fit of these data results in a  $K_d$  of  $16 \pm 5 \mu\text{M}$ , indicating that the interaction between calcium ions and the nanospheres is quite strong. The  $K_d$  for the interaction of calcium with the molten globule state of BLA is  $\sim 1 \text{ mM}$  (31). Consequently, calcium ions cannot be present in the calcium binding site of the adsorbed BLA molecules, but are instead adsorbed on the surface of the nanospheres.

## DISCUSSION

In this study we show that the kinetics of adsorption-induced conformational changes can be determined with stopped-flow fluorescence spectroscopy. The main unfolding event is remarkably fast and results in a partially unfolded state. The spectroscopic observations made here for the adsorbed state of BLA, *i.e.* tertiary structure loss but persistent secondary structure, are known to be characteristic for a molten globule state, which is an intermediate in the folding pathway of BLA in solution (13, 32). A molten globule state has increased exposure of



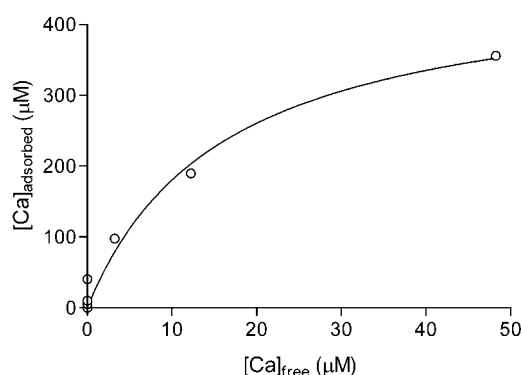


FIG. 9. **Adsorption isotherm of calcium on polystyrene nanospheres in a 10 mM Tris/HCl buffer at pH 7.5 after 20 h of adsorption.** Circles, data; line, fit. The concentration of the nanospheres is 80 nm. Each data point is the average of three measurements. The filtrate (Amicon 50-kDa ultrafiltration) was analyzed for calcium content by ICP-OES. The data are fitted with a Langmuir equation:  $[Ca]_{\text{adsorbed}} = (B*[Ca]_{\text{free}})/(K_d + [Ca]_{\text{free}})$ , resulting in a  $K_d$  of  $16 \pm 5 \mu\text{M}$ , a  $B$  value of  $4.7 \times 10^2 \pm 0.6 \times 10^2 \mu\text{M}$ , and an  $R^2$  value of 0.98. In the latter equation,  $B$  is the plateau value of adsorption, and  $K_d$  is the dissociation constant.

hydrophobic groups, which can be measured by hydrophobic fluorescence probes like 1-anilinoanthracene-8-sulfonate (ANS) (33). In addition, it has been shown that adsorption of BLA on a hydrophobic surface like polystyrene occurs via hydrophobic interactions (34–36). Our observation that adsorbed BLA has a molten globule conformation with exposed hydrophobic groups supports the latter. Furthermore, we show that calcium cannot be present in adsorbed BLA, which further supports the presence of an adsorbed molten globule state of BLA.

**Orientation of BLA Molecules on the Nanosphere Surface—**Despite the importance of hydrophobic interactions for BLA adsorption, it is not likely that all four tryptophans of adsorbed BLA are in close contact with the hydrophobic polystyrene surface (dielectric constant = 2.6). This is supported by the observed red shift of the fluorescence emission maximum, indicating that one or more Trp residues are more exposed to the solvent (Fig. 2). A number of hydrophobic residues must be in close contact with the hydrophobic polystyrene surface for binding through hydrophobic interactions to occur. The preference of these residues for a position close to the hydrophobic polystyrene surface results in a specific conformation of the BLA molecule on the surface, which affects the environment of the tryptophan residues. Investigation of the crystal structure of BLA can give us a clue about the preferred orientation of BLA on a hydrophobic interface. At pH 7.5, both BLA and the nanosphere surface have a net negative charge. The negative charge on the surface of a BLA molecule is concentrated in a patch near the calcium binding site. This patch includes aspartic acid residues 14, 63, 64, 78, 82, 83, 84, and 87 and glutamic acid residues 1, 7, and 11. The most likely orientation of a BLA molecule approaching a negatively charged interface would thus be with the negative patch facing toward the solution, pointing away from the interface. Note that this orientation would not hinder calcium release of BLA in the adsorbed state. In addition, a possible candidate for orientation of BLA by hydrophobic interactions is the hydrophobic patch formed by the “flexible loop” (residues 105–111) and the adjacent aromatic cluster I (residues 31, 32, 117, and 118) (37). Both hydrophobic regions are thought to be involved in binding of BLA to galactosyl transferase, resulting in the enzyme complex lactose synthase. The hydrophobic patch is on the opposite side of the calcium binding site, thereby enabling orientation by both electrostatic and hydrophobic interactions.

**The Scheme for the Adsorption of BLA on Polystyrene Nanospheres—**In Fig. 8 a scheme is proposed for the adsorption and unfolding processes that play a role during adsorption of BLA on the polystyrene nanospheres. In solution the native state of BLA ( $N_s$ ) is in equilibrium with an intermediate state ( $I_s$ ), which is generally known to be a molten globule state. Two routes are depicted that lead from the start situation, native BLA ( $N_s$ ), to the end situation of adsorbed and partially unfolded BLA ( $D_a$ ). The first route involves the adsorbed native state  $N_a$ , whereas the second route includes the  $I_s$  state. An argument in favor of the second route is the hydrophobic nature of the intermediate state of  $\alpha$ -lactalbumin in solution, which would more easily adsorb onto a hydrophobic surface than the native state does. However, the population of the intermediate state is very low in solution, so the chance that the intermediate state comes in proximity of a nanosphere is very small. The rate of GdnHCl-induced unfolding of native BLA to the intermediate state  $I_s$  is about  $0.001 \text{ s}^{-1}$  when extrapolated to 0 M GdnHCl (31). If the adsorption and subsequent unfolding route would proceed via  $I_s$ , the observed rate of unfolding would be affected by this step. Because the rate constant we find is four orders of magnitude larger, the latter route can be excluded as indicated by the shaded areas in the scheme (Fig. 8).

The most likely route for BLA adsorption involves the adsorbed native state  $N_a$ . After adsorption, the native state is partially unfolded to a denatured state  $D_a$ . The denatured state is characterized by conserved secondary structure, lost tertiary structure, and loss of the calcium ion. The adsorbed denatured state has the typical characteristics of the molten globule state of BLA ( $I_s$ ), which is an intermediate during the folding of the protein. The adsorption-induced partial unfolding of  $N_a$  occurs rapidly as 50–70% of the fluorescence intensity increase already occurs in the 15-ms dead time of the experiment and a further 30–35% increase has a rate constant of  $38\text{--}74 \text{ s}^{-1}$ . It is interesting to compare the adsorption-induced unfolding rates with the rate of BLA unfolding induced by common denaturants like guanidinium hydrochloride (GdnHCl). The rate of GdnHCl-induced unfolding of native BLA at 5.6 M GdnHCl is about  $4 \text{ s}^{-1}$  (31). Our observation is similar: When BLA is unfolded in 5.2 M GdnHCl the fluorescence unfolding trace could be fitted to a single-exponential function with a rate constant of  $2.4 \pm 0.2 \text{ s}^{-1}$ . The unfolding of BLA on polystyrene nanospheres has a remarkably fast rate of  $50 \text{ s}^{-1}$  or higher as shown by our experiments. The adsorbed and partially denatured state has stable interactions with the polystyrene interface and desorption hardly occurs. The polystyrene/water interface thereby acts like a local sink, or more probably like a series of local sinks, that stabilize folding intermediates located somewhere in the folding energy landscape between the native state and the array of completely unfolded species of BLA.

It is unlikely that a single population of denatured species with identical conformational properties exists in the adsorbed state. There are many factors involved that make the adsorption process different for individual molecules. First, the homogeneity of the polystyrene/water interface does not need to be perfect; one can think of physical irregularities and unequal distribution of charged surface groups. The first arriving BLA molecules will predominantly occupy sites that lead to the lowest free energy of the adsorbed molecules. Second, when the interface becomes more crowded other factors become important, for instance protein-protein interactions. Although the major unfolding phase with rate  $k_1$  is most probably related to the transition of the native state to the partly denatured state, it is much harder to assign the slower phases  $k_2$  and  $k_3$ . Most probably the latter phases are caused by subsequent local

unfolding processes of the partly denatured, adsorbed BLA molecules, which do not affect the secondary structure and influence only the Trp environments. It is also possible that BLA molecules that adsorb when the interface is almost covered (but still in the dead time of the experiment) unfold much slower because of steric hindrance or protein-protein interactions. However, because  $k_2$  does not depend on the BLA concentration, the latter possibility seems to be unlikely.

As indicated in the proposed scheme, it is further assumed that the desorption rate of the partly denatured, adsorbed BLA ( $D_a$ ) is negligible. Upon adsorption of a protein molecule there will most likely be a number of interacting sites between the protein molecule and the interface (2). As is known from protein chromatography, a site density on a chromatographic support that is too high can lead to irreversible adsorption of the protein. Our experiments show that desorption of BLA molecules does not occur upon washing with buffer, even after 24 h. Completely unfolded adsorbed species are omitted from the scheme for adsorption of BLA, because the fluorescence maximum indicates that the adsorbed BLA molecules are not completely unfolded.

**Concluding Remarks**—Unlike heat-, pressure-, or denaturant-induced protein unfolding, adsorption-induced unfolding of proteins has received little attention. An important cause for the latter is the impossibility to acquire classic equilibrium unfolding curves like those routinely obtained for denaturant-induced protein unfolding. In fact, adsorption under our conditions is a nearly irreversible thermodynamic process. Consequently, a thermodynamic analysis of the data similar to the one applied to classic equilibrium unfolding data is impossible. As opposed to protein unfolding by high concentrations of a conventional denaturant, adsorption-induced protein unfolding, as shown here for BLA, can already be achieved by the addition of only 5 nM polystyrene nanospheres, which corresponds to about 2.5  $\mu$ M interaction sites. It is thus a major challenge to obtain detailed information on the molecular level about adsorption-induced protein unfolding. The importance of the phenomenon of protein adsorption in everyday life further warrants the investigation of adsorption-induced protein unfolding.

Here it is shown by tryptophan fluorescence spectroscopy, by far-UV and near-UV CD spectroscopy, and by the observation of the release of calcium that adsorption of bovine  $\alpha$ -lactalbumin on polystyrene nanospheres leads to a denatured state of the BLA molecule, which has the characteristics of a molten globule. The time-dependent conformational changes observed upon adsorption of BLA are not affected by diffusion or association processes. The rate of partial unfolding of adsorbed BLA molecules is remarkably fast (38–74 s<sup>-1</sup>) and exceeds significantly the rate of BLA unfolding induced by the addition of a high concentration of denaturant, *i.e.* 5.4 M GdnHCl.

The results presented here contribute to the understanding of protein unfolding that is induced by the interaction of a protein with an interface. To obtain further information about

the conformational characteristics of adsorbed BLA, we envision hydrogen-deuterium exchange experiments on adsorbed BLA. The exchange of labile hydrogen atoms of adsorbed BLA will then be monitored by NMR in the desorbed state of the protein.

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